Improvement of RNA Aptamer Activity against Myasthenic Autoantibodies by Extended Sequence Selection

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Myasthenia gravis (MG) is mainly engendered by autoantibodies directed against acetylcholine receptors (AChRs) located in the postsynaptic muscle cell membrane. Previously, we isolated an RNA aptamer with 2'-amino pyrimidines using in vitro selection techniques that acted as a decoy against both a rat monoclonal antibody called mAb198, which recognizes the main immunogenic region on the AChR, and patient autoantibodies with MG (1). However, low affinity of this RNA to mAb198 relative to that of AChR might limit potential of the RNA as an inhibitor of the autoantibodies. To improve decoy activity of the RNA aptamer against autoantibodies, here we employed in vitro selection methods with RNA libraries containing extra random nucleotides extended to the 3' end of previously selected RNA sequences. RNAs isolated in this study showed significant increases in the binding affinities to mAb198 as well as bioactivities protecting AChRs on human cells from both mAb198 and patient autoantibodies, compared with the previous RNA aptamers. These results have important implications for the development of antigen-specific modulation of autoimmune diseases including MG.

Key Words: in vitro selection; RNA aptamer; myasthenia gravis; acetylcholine receptor; autoantibody.

Myasthenia gravis (MG) is a human neuromuscular autoimmune disease associated with muscular weakness and fatigability. The symptoms of MG are mainly due to autoantibody-mediated reduction in nicotinic acetylcholine receptors (AChRs) available on the skeletal muscle at neuromuscular junctions (2, 3). Immunization with purified AChR or passive transfer of anti-AChR antibodies have been reported to induce experimental autoimmune myasthenia gravis (EAMG) in animals (3). The majority of anti-AChR antibodies found in both human patients with MG and rats with EAMG are directed against the main immunogenic region (MIR) on the extracellular domain of the AChR α-subunit (4, 5). Because the current MG therapy including general immunosuppression often results in toxic side effects (2), a more specific and efficient therapy for MG is required.

Since the pathogenesis of MG is mainly mediated by autoantibodies to AChRs, much effort has been directed toward the development of small molecules, such as anti-AChR Fab fragments or peptide mimotopes of AChR, that inhibit binding of these pathogenic autoantibodies to muscle AChRs (6–8). However, the isolation of short peptides that bind to target proteins with high affinity has been difficult mainly due to their unstable folded structure (9). In contrast, short RNA molecules can form a fairly stable and unique tertiary structure via intramolecular base-pairing (9, 10). Moreover, short RNA aptamer molecules have been isolated to bind several proteins including non-RNA binding proteins such as antibodies with high affinity and specificity using in vitro selection techniques from random RNA libraries (1, 11–14). It has been also reported that certain RNA molecules selected in vitro can inhibit the physiological functions known to be associated with the target proteins in animal models (15). Thus, these results suggest that RNA molecules could be alternative candidates for specific and effective inhibitors of MG autoantibodies.

Previously, we employed in vitro selection methods and isolated a nuclease-resistant RNA aptamer with 2'-amino-modified pyrimidines that can bind a rat monoclonal antibody, called mAb198, that is specific to the MIR on human AChR, and patient autoantibodies with MG (1). This RNA has been shown to act as a decoy to block the antibodies from their specific receptor, AChR, in human cells. However, more potent RNA inhibitors against MG autoantibodies might be needed because of the relatively low efficacy of the selected...
RNA (SE RNA) as an autoantibody inhibitor (IC\(_{50}\) = 5–10 \(\mu\)M, 1) compared with that of anti-AChR Fab (IC\(_{50}\) of less than 0.1 \(\mu\)M, 16). This limitation might be due to the relative low affinity of the RNA for mAb198 (K\(_d\) = 5; 60 nM) compared to that of the human AChR for the antibody (K\(_d\) = 22 nM) (1).

Accordingly, to develop more effective RNA decoys, we employed an RNA combinatorial library with extra random nucleotides extended to the 3’ end of previously selected RNA sequences and in vitro selection techniques. The RNA aptamers selected here were shown to bind mAb198 more avidly than not only the previously selected RNA aptamers but also AChR. Moreover, they inhibited both mAb198 and MG patient autoantibodies from down-modulating AChR expression on human cells more effectively than the previous RNAs.

**MATERIALS AND METHODS**

Antibodies, AChR, and cells. Rat mAb198 was kindly provided by Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). Serum samples from patients with MG (batch No. 2051) were purchased from The Binding Site (San Diego, CA). The major extracellular domain of the human AChR α-subunit (amino acids 1–210) was a gift from Sohel Talib (Gencell, Santa Clara, CA). TE671 human medulloblastoma cell lines (ATCC 8805-CRL) of rhabdomyosarcoma origin (17) express receptors for acetylcholine apparently identical to human AChR (18) and were used in acetylcholine receptor down-modulation experiments.

Selection procedure. Extended RNA library was generated by in vitro transcription of synthetic DNA templates with extra 20 random nucleotides (nts) attached to the 3’ end of sequences selected previously against mAb198 (1) using 2’-deoxy-2’-amino CTP and UTP (Amersham, Arlington Heights, IL) and normal GTP, ATP, and T7 RNA polymerase. The sequence of resulting RNA library is 5’-GGGAGAGCGGAAGCGUGCUGGGCCGGAGGUUAGCUUGCCAUGGCAAGCAGGGCGCCACGGACCN\(_{20}\)CAUAACCCAGAGGUGAUGGAUCCCCCC-3’ (Amersham, Arlington Heights, IL). The major extracellular domain of the human AChR α-subunit (amino acids 1–210) was a gift from Sohel Talib (Gencell, Santa Clara, CA). TE671 human medulloblastoma cell lines (ATCC 8805-CRL) of rhabdomyosarcoma origin (17) express receptors for acetylcholine apparently identical to human AChR (18) and were used in acetylcholine receptor down-modulation experiments.

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cataway, NJ) and discarded to remove RNAs that bind to the constant region of antibodies or adhere nonspecifically to the beads. The preleared supernatant was transferred to a new tube and further incubated with 2.5 μg of rat mAb198 for 30 min at room temperature. MAb198–RNA complexes were immunoprecipitated and washed 5 times with 0.5 ml of the binding buffer. RNAs were recovered, amplified, and used in the next rounds of selection. In the previous experiment (1), 10 more rounds of selection were repeated using 2.5 μg of mAb198. In contrast, here we performed subsequent selections with more stringent conditions by reducing mAb198 concentration by 5- to 10-fold at every 3 round: 2.5 μg (rounds 1–3), 0.5 μg (rounds 4–6), 0.05 μg (rounds 7–9), or 0.005 μg (rounds 10–12). After 12 rounds of selection, the amplified DNA was cloned and sequenced, and the finally selected RNAs were analyzed as previously described (1, 12).

Analysis of selected RNAs. Newly selected RNAs with 2′-deoxy-2′-amino pyrimidines were internally radiolabeled and isolated as described (1, 19). Purified RNAs were incubated with different antibodies as described above. The antibody–RNA complexes were analyzed on a 4% nondenaturing polyacrylamide gel containing 10 mM MgCl2 and 2% glycerol for gel shift analysis. Alternatively, antibody–RNA complexes were immunoprecipitated with protein G-Sepharose beads, and bound RNAs eluted from the pellets. RNAs were then analyzed on a 6% polyacrylamide gel with urea.

Assay of AChR down-modulation. AChR down-modulation was monitored as described (1). MAb198 (5 nM) or autoantibodies from patients with MG were preincubated in 90 μl of media (DMEM) containing 10 mM MgCl2, 1% BSA, and 10% FBS with or without RNA for 30 min at room temperature. These mixtures were then added to TE671 cells in the presence of 40 μg/ml of cycloheximide and incubated at 37° for 4 h. Next, 10 μl of media with 50 nM of 125I-radiolabeled α-bungarotoxin (α-Bgt) was added and incubated for three more hours. The cells were then washed, and the bound α-Bgt was quantitated using a γ 5000 counter (Packard Instruments, Meriden, CT). Incubation of TE671 cells with 5 nM of mAb198 alone reproducibly reduced AChR expression of the cell surface by approximately 50% (data not shown). This level of antigenic down-modulation was considered 100%, and other values were normalized to this amount. For antigenic down-modulation experiments with patient autoantibodies, patient serum was added to TE671 cells at concentration determined to engender similar levels of antigenic down-modulation as 5 nM of mAb198: 1 μl of 1:2 dilution of patient sera (data not shown).

RESULTS AND DISCUSSION

In Vitro Selection of Extended RNA Sequences Specific to mAb198

An RNA library of approximately 1013 different molecules was generated with every pyrimidine substituted at its 2′ position by amino group. Changing the 2′ position on the pyrimidines of an RNA from hydroxyl group to amino group extremely increased the stability in serum (1, 12, 20). Each molecule in the library contained an extra 20-nucleotide (nt) long random sequences extended to the 3′ end of previously selected sequences against mAb198 (1). To isolate higher-affinity RNAs that bound to mAb198, we employed in vitro selection method using the RNA library with more stringent conditions than the previous experiment (1) as described under Materials and Methods. After 12 rounds of selection, the bound RNAs were amplified by RT-PCR and the resulting cDNAs cloned. Eighteen different clones were sequenced, and then five different RNAs were selected and found in multiple clones (Fig. 1A). To see the mAb198-specific binding of the RNAs containing newly selected sequences that are extended to the 3′ end of previously selected sequences (Ex SE RNA), a gel retardation experiment was performed with internally radiolabeled Ex SE RNAs (Fig. 1B). Every Ex SE RNA efficiently formed a shifted nucleoprotein complex with mAb198 (Fig. 1B, lane b), but not with normal rat IgGs (Fig. 1B, lane a).

High-Affinity Binding of the Selected RNAs to mAb198

The binding affinity of the Ex SE RNAs to mAb198 was determined by a gel retardation assay with trace amounts of radiolabeled RNAs and increasing amounts of mAb198 (Fig. 2). RNA library containing 40-nt-long random sequences that was used for the previous selection experiment against mAb198 (1, Library RNA) was shown to have little affinity to mAb198 even in the highest concentration of the antibody. However, SE RNA selected previously with 2′-amino pyrimidines (2′-NH2 SE RNA) showed high affinity with about 60 nM of the equilibrium dissociation constant (Kd) as observed in the previous experiments (1), RNA library containing extended 20-nt-long random sequences which was utilized in this study (Ex Library RNA)
showed similar affinity to 2'-NH₂ SE RNA. In contrast, the Kₐ of 6–30 nM, affinity that was more than 2- to 10-fold stronger than 2'-NH₂ SE RNA, was detected for the Ex SE RNAs isolated in this study. Hence, newly selected Ex SE RNAs bind much more avidly to mAb198 than 2'-NH₂ SE RNA. Especially, since Ex SE RNA clone V was found to have the highest affinity (Kₐ approximately 6 nM), we utilized it for further characterizations. The binding affinity of Ex SE RNA V was also greater than that of human AChR (Kₐ about 21.6 ± 6.6 nM, 7).

As a working model, the most stable secondary structure of Ex SE RNA V was predicted using the MULFOLD program (21) as shown in Fig. 3. In this folded structure, the 20-nt-long sequences, derived from the randomized region of the Ex Library RNA, are predicted to form base-pairing interactions with the defined sequences that flank both previously selected sequences and the random region in the library RNA. Interestingly, the newly selected RNA sequences did not disturb the secondary structure of minimal binding domain of 2'-NH₂ SE RNA encompassing +20 to +64 nt which was identified in the previous study (1). Therefore, newly selected sequences could most likely stabilize the structure of Ex SE RNA that is required for binding to mAb198, and thus increase the binding affinity. Other selected RNA clones showed similar pattern of predicted secondary structure to clone V. Namely, newly selected sequences base-paired with the defined flanking sequences as described here, but not the minimal sequences for mAb198 binding (data not shown).

Selected RNAs Block mAb198 from Binding to and Down-Modulation of AChR on Human Cells

To determine the binding site of mAb198 to Ex SE RNA aptamer, excess amount of purified ectodomain of
the human AChR α-subunit was incubated with the antibody in the presence of radiolabeled competitor Ex SE RNA (Fig. 4). The purified human AChR α-subunit used in this study that encompasses the major extracellular domain was reported to direct the majority of anti-AChR antibodies in MG patients and induce EAMG in rats (4, 5, 22). Incubated reaction mixtures were immunoprecipitated and analyzed for the evaluation of Ex SE RNA V binding activity to mAb198. The specific interaction between Ex SE RNA V and mAb198 can be blocked by the presence of human AChR in a concentration-dependent manner (lanes 6 and 7). Therefore, Ex SE RNA V appears to bind mAb198 at or near the antibody’s combining site.

Once we had ascertained that Ex SE RNA V avidly and specifically bound to mAb198 at its combining site, we wanted to determine if the RNA could inhibit mAb198 from binding to AChR on human cells and protect cells from the biological effects associated with the antibody in culture. Incubation of human TE671 cells with mAb198 resulted in a dose- and time-dependent decrease in the number of AChRs on the cell surface (data not shown, 16), which is termed antigenic down-modulation. To determine if Ex SE RNA V could protect cells from mAb198 binding and inhibit antibody-mediated down-modulation of AChR on human cells, TE671 cells were incubated with mAb198 in the presence or absence of RNAs (Fig. 5). Degradation of AChRs induced by mAb198 was easily monitored by observing the amount of I\(^{125}\) α-bungarotoxin (α-Bgt) that can bind AChRs remaining on the cells after treatment of the antibody as previously described (1). While a nonspecific RNA competitor, such as the Library RNA with 40-nt-long random sequences, could not inhibit mAb198-mediated down-modulation of AChR expression, Ex SE RNA V protected the TE671 cells from mAb198 and effectively inhibited antigenic down-modulation by up to 80% (Figs. 5A and 5B). Furthermore, Ex SE RNA-mediated inhibition was dose-dependent with an IC\(_{50}\) of about 0.4 μM (Fig. 5B). Inhibition of antigenic modulation by the Ex SE RNA V selected in this study was about 10-fold more efficient...
than that by 2'-NH$_2$ SE RNA (IC$_{50} = \sim 4$ $\mu$M, Fig. 5B), indicating that increase in the binding affinity of SE RNA to mAb198 can improve the bioactivity of the RNA aptamer to inhibit the antibody effects.

Alternative way to improve RNA aptamer activity by increase in its binding affinity to target molecules might be selecting RNAs with 2'-fluoro pyrimidines in place of 2'-amino pyrimidines because the extreme thermostability and more rigid structure could be found in RNA with 2'-fluoro groups (23). However, the selection of RNA with 2'-fluoro groups (2'-F SE RNA) against mAb198 was reported to increase the binding activity (K$_d = \sim 25$ nM) and the bioactivity (IC$_{50} = \sim 2.4$ $\mu$M) of the RNA only by 2-fold, compared to 2'-NH$_2$ SE RNA, in our recent study (24).

Selected RNAs Inhibit Autoantibody-Mediated Down-Modulation of AChR on Human Cells

Since patient sera with MG often contains autoantibodies that recognize the same MIR of human AChR as mAb198 (5) and Ex SE RNA V cross-reacts with patient’s anti-AChR autoantibodies tested here using immunoprecipitation experiment (data not shown), Ex SE RNA aptamer might resemble the common epitope of the MIR on AChR that was recognized by both mAb198 and MG autoantibodies. Moreover, most pathologically relevant autoantibodies from MG patients appear to bind the MIR on AChR (3). Therefore, the Ex SE RNA could protect cells from a significant fraction of autoantibodies and act as a blocking agent for MG patient sera-mediated receptor down-modulation in human cells. To explore this possibility, the ability of Ex SE RNA V to protect cells from the MG patients’ autoimmune sera was evaluated (Fig. 6). Incubation of TE671 cells with the patient sera induced time- and dose-dependent antigenic down-modulation of AChR (data not shown, 1). Addition of Ex SE RNA, but not nonspecific RNA such as Library RNA, blocked some fraction of the patient’s autoantibodies from binding to and, hence, down-modulation of AChR on TE671 cells by approximately 65% (Figs. 6A and 6B). Furthermore, Ex SE RNA-mediated inhibition was dose-dependent with an IC$_{50}$ of about 2 $\mu$M, a value 5-fold better than that of 2'-NH$_2$ SE RNA (IC$_{50} = \sim 10$ $\mu$M, Fig. 6B) and 2'-F SE RNA (IC$_{50} = \sim 9$ $\mu$M, 24). Therefore, this Ex SE RNA can protect a significant fraction of the AChRs present on cells from the autoantibodies in the sera of MG patient more efficiently than the previous RNA aptamers.

In this study, we developed new selection methods by employing RNA libraries containing extra random nucleotides extended to the 3' end of RNA sequences isolated previously. The binding activity of the newly selected RNA aptamers to mAb198 was effectively improved by up to 10-fold. This might be because the sequences selected from randomized region confer stability to the RNA structure of mAb198 binding domain by base-pairing with flanking sequences. Furthermore, the bioactivity to inhibit the deleterious effects of autoantibodies was also noticeably increased by more than 5- to 10-fold.

These results together with the previous observations (1) suggest that RNAs selected in vitro could be useful reagents to inhibit the autoantibody-mediated...
immune responses in MG patients. However, heterogeneous populations of anti-AChR autoantibodies present in patient sera (25) will probably limit the utility of the RNAs. Therefore, RNAs to block a greater fraction of autoantibodies found in MG patients should be developed to be therapeutically valuable. Such RNAs could be generated by selecting RNAs against additional anti-MIR monoclonal antibodies or a pool of autoantibodies in MG patients. If a combination of such RNA aptamers prevents the binding of a major fraction of autoantibodies in serum of EAMG rats or MG patients, the RNAs then can be potentially applied as lead compounds for specific agents of MG therapy.

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